9-2006

A New Greenhouse Method to Assay Soybean Resistance to Brown Stem Rot

G. A. Tabor
Iowa State University

S. R. Cianzio
Iowa State University, scianzio@iastate.edu

G. L. Tylka
Iowa State University, gltylka@iastate.edu

R. Roorda
Iowa State University

C. R. Bronson
Iowa State University

A New Greenhouse Method to Assay Soybean Resistance to Brown Stem Rot

G. M. Tabor, Department of Plant Pathology, Iowa State University, Ames 50011; **S. R. Cianzio,** Department of Agronomy, Iowa State University, Ames 50011; and **G. L. Tylka, R. Roorda,** and **C. R. Bronson,** Department of Plant Pathology, Iowa State University, Ames 50011

ABSTRACT

Tabor, G. M., Cianzio, S. R., Tylka, G. L., Roorda, R., and Bronson, C. R. 2006. A new greenhouse method to assay soybean resistance to brown stem rot. Plant Dis. 90:1186-1194.

Greenhouse, growth chamber, and field experiments were conducted to develop a method to assess resistance of soybeans to *Cadophora gregata* (*Phialophora gregata*), causal agent of brown stem rot (BSR). In the new method, *C. gregata* is introduced at the base of the stems of 2-week-old soybeans, and the presence of the fungus is assessed in the tips of the stems 5 weeks later. To test the effectiveness of the method, two populations of soybeans and 10 checks were inoculated at the stem base and then assayed for fungal colonization of the stem tips, percentage of symptomatic leaflets, and percent internal stem length discolored. The lines also were planted in naturally infested fields to assess for percent internal stem length discolored, and were tested for the presence/absence of a BSR-resistant molecular marker. Greenhouse, field, and molecular marker data were compared. Linear regression analysis suggested that percentage of plants with colonized stem tips explained 41 to 64% of the variability ($P < 0.0001$) in percent stem length discolored in the field and 58 to 85% of the variability ($P < 0.0001$) in molecular marker data for BSR resistance. Percent stem length discolored assessed in the greenhouse had the lowest correlation with percent stem length discolored in the field and with the molecular marker. Of three incubation temperatures tested, 22°C was the most conducive for distinguishing resistant/susceptible soybeans using the colonization method.

Brown stem rot (BSR) is an economically important disease of soybeans (*Glycine max* (L.) Merr.) in the north-central United States. It is caused by the vascular pathogen *Cadophora gregata* Harrington & McNew (3) (*Phialophora gregata*) (Allington & D.W. Chamberlain) W. Gams (AC) (1). BSR is prevalent in 68 to 73% of the soybean fields in Illinois, Iowa, and Minnesota (18). The currently recommended management strategy is use of BSR-resistant soybean cultivars combined with rotation to nonhost crops (19). *C. gregata* in the Midwest is composed of two genotypes (4) that differ in their aggressiveness and ability to cause BSR symptoms on certain soybean genotypes (2,4,5). Genotype A is generally recognized as the more aggressive based on symptom expression.

Selection and breeding for BSR-resistant cultivars relies heavily on field screenings on BSR-infested soil. Because of the effects of factors such as unfavorable weather, insufficient inoculum and/or uneven inoculum distribution in the soil, and presence of other pathogens in the field that can break resistance to BSR (13), field screening must be done at multiple locations over several seasons to obtain reliable results (9). This is both expensive and time-consuming.

Alternatives to field screening are molecular markers (6) and assessments of stem and/or foliar symptoms in inoculated plants grown in greenhouse or growth chamber conditions (7,10,11,12,14,17). Molecular markers are useful only if the source of resistance is known and the resistance co-segregates with the marker(s). Greenhouse and growth chamber screens can be problematic as well and often give unreliable results due to inconsistent symptom expression (12,14). In addition, BSR symptoms are measured subjectively and therefore are subject to experimenter bias. A screening method that does not depend on molecular markers or symptom assessment will be a valuable addition to the pool of BSR assays.

Recent research indicates that BSR resistance is expressed in the stems of resistant soybeans and that this resistance can be detected as a reduction in the height of fungal colonization in resistant stems (14). This research demonstrated that the fungus advances with the growing tip in susceptible soybean genotypes and lags behind in resistant genotypes after introduction of the fungus into the base of 2-week-old plants (14). This difference in the movement of the fungus in stems of resistant and susceptible soybean genotypes might be exploited to develop a screening method that involves assaying for the presence of the fungus in the tips of inoculated plants. Such a screening method would not depend on symptom expression and therefore may be less subject to experimenter bias.

The specific objectives of the research reported herein are (i) to develop a fungal-colonization assay for BSR resistance and compare the new assay with symptom-based assays and molecular marker assays, and (ii) to test the effect of incubation temperature on the reliability of the new assay. During the course of the study, we also determined the mode of inheritance of BSR resistance in two inbred populations.

MATERIALS AND METHODS

Experiment names. A total of 11 experiments were conducted. For clarity, these are identified by a combination of numbers and letters. Four field experiments were conducted in 2000 and 2001 at two locations in Iowa to test two soybean breeding populations for BSR resistance using a standard BSR-resistance screening method. Experiments 1A and 2A were conducted in Ames, IA, in 2000 and 2001, respectively. Experiments 1B and 2B were conducted in Mason City (NIACC area), IA, in 2000 and 2001, respectively. The same soybean lines in the above experiments were tested for BSR resistance in a greenhouse (experiments 3A, 3B, 4A, and 4B) using fungal-colonization, foliar symptom, and internal stem discoloration assays. Also, two growth chamber experiments (5A and 5B) were conducted to test the effect of three temperature regimes on the fungal-colonization assay. A molecular marker associated with BSR resistance was assessed in experiment 6.

Soybean genotypes. The soybean lines tested in field, greenhouse, and molecular marker experiments (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, and 6) were two populations developed for the study, AX14853 and AX15633. The parents of each population and check cultivars (five BSR-resistant and five BSR-susceptible) were also included in the experiments. Population 1 (AX14853) was obtained from the cross of a BSR-susceptible genotype A95-581022 with a BSR-resistant genotype A96-597011. Population 2 (AX15633) was developed from the cross of Pioneer 9233, a BSR-susceptible genotype, to a BSR-resistant genotype, A95-682026. Genotypes A96-597011 and A95-682026 were advanced experimental lines from Iowa State University developed for BSR resistance from crosses which derived their resistance from

Corresponding author: G. M. Tabor
E-mail: gtabor@iastate.edu

Accepted for publication 27 April 2006.

DOI: 10.1094/PD-90-1186

cultivar BSR 101. The cultivar BSR 101 (15) was developed by Iowa State University and traces its resistance to PI 84946-2. Pioneer 9233 is a high-yielding line from Pioneer Hi-Bred International. The crosses, population development, and inbreeding to obtain lines for the study were conducted at the Isabela Substation of the University of Puerto Rico, Isabela, Puerto Rico. Fifty random lines from each population were used in the above experiments. Lines in population 1 were in the F_6 generation (F_6-derived lines), and lines in population 2 were in the F_5 (F_5-derived lines) generation. In experiments 5A and

Table 1. Brown stem rot resistance ratings of two breeding populations using field and various greenhouse assays

Population 1[t]						Population 2[t]					
	Field[u]	Greenhouse[v]					Field[u]	Greenhouse[w]			
Entry	% Stem length discolored	% Stem length discolored	% Symptomatic leaflets	% Stem tips colonized	Molecular marker[x]	Entry	% Stem length discolored	% Stem length discolored	% Symptomatic leaflets	% Stem tips colonized	Molecular marker[x]
Progeny[y]						Progeny[y]					
151018	19	64	11	6	R	152011	5	59	20	0	R
150079	8	63	17	7	R	152021	12	52	7	0	R
151007	14	73	17	7	R	152038	6	40	26	0	R
151049	14	71	22	7	R	152088	9	34	12	0	R
151012	10	59	15	18	R	152096	6	56	21	0	R
150023	11	79	25	20	R	151053	6	49	11	5	R
150017	15	87	33	25	R	152029	10	40	14	5	R
150057	10	84	34	25	R	152056	9	45	17	5	R
150074	11	55	20	25	R	152060	9	44	11	5	R
150005	15	64	22	27	R	152092	16	53	14	5	R
151045	9	81	31	27	R	152003	4	49	16	10	R
151035	15	66	25	29	RS	152005	5	60	18	10	R
150042	17	79	24	31	R	152013	10	51	30	10	R
150045	13	72	28	31	R	152031	9	47	14	10	R
150072	8	65	11	31	R	151093	10	70	33	15	RS
150008	9	42	8	33	R	152035	12	51	23	15	R
150063	17	76	33	33	R	152073	12	56	28	15	R
151008	19	74	30	33	R	152086	8	29	18	15	R
151009	10	85	15	36	R	151082	16	56	48	20	RS
150039	10	89	14	38	R	152018	6	51	28	20	R
151022	4	53	5	38	R	152079	8	44	32	20	R
151002	12	71	25	40	R	151060	10	45	32	22	RS
150096	11	83	12	42	R	151085	16	57	37	26	RS
151024	16	83	28	42	R	152082	15	40	31	30	RS
151048	16	83	25	44	RS	152074	22	51	58	32	S
150046	20	72	37	46	S	152098	15	34	33	35	S
150058	15	89	40	46	RS	151091	13	56	46	50	RS
150091	19	92	31	46	R	151097	16	81	64	50	RS
150024	8	63	11	47	R	151075	34	64	75	55	S
151025	8	72	17	47	R	151087	26	70	60	55	RS
150068	11	65	39	50	R	152001	10	62	65	55	S
150085	34	85	55	50	S	152032	22	65	74	65	S
150067	17	82	29	53	RS	152042	23	63	77	65	S
150059	15	87	45	54	R	152069	22	67	45	65	S
150095	14	72	24	60	R	152076	24	72	74	65	S
151044	28	82	25	60	S	151081	25	78	74	68	S
150028	26	65	22	64	S	152065	27	69	71	68	S
150051	37	88	42	64	S	152089	25	54	70	68	S
150097	38	94	50	64	S	151066	24	66	63	70	S
150055	27	78	21	67	S	151099	29	70	75	70	S
151026	14	77	22	67	R	152046	22	58	60	70	S
150011	39	92	49	69	S	152081	20	58	63	70	S
150094	24	73	25	69	S	152087	19	69	84	70	S
151039	24	92	40	69	S	151071	31	74	86	74	S
150088	22	73	34	75	S	151077	29	71	69	75	S
151017	22	81	37	75	S	152097	16	48	59	75	S
150071	27	80	30	79	S	151080	28	69	78	79	S
150090	22	83	30	82	S	152049	17	58	70	80	S
150036	27	77	39	86	S	152055	19	61	67	80	S
151038	34	83	35	90	S	152083	20	64	71	80	S
LSD[z]	11	19	14	30		LSD[z]	9	17	17	24	

[t] Data were analyzed using GLM in which year, location, and replications (for field experiments) and replications and experiments (for greenhouse experiments) are treated as random effects. Population 1 (AX14853) was obtained from the cross of a BSR-susceptible genotype A95-581022 with a BSR-resistant genotype A96-597011. Population 2 (AX15633) was developed from the cross of Pioneer 9233, a BSR-susceptible genotype, to a BSR-resistant genotype, A95-682026.

[u] Numbers are means from analysis of combined experiments 1A, 1B, 2A, and 2B, each with two replications, and there are 10 plants in each replication.

[v] Numbers are means from analysis of combined experiments 3A and 3B, seven and nine replications in each, respectively, and there is one plant in each replication.

[w] Numbers are means from combined experiments 4A and 4B, 10 replications in each, and there is one plant in each replication.

[x] R = BSR-resistant marker and S = BSR-susceptible marker.

[y] Progeny are sorted by % stem tips colonized in ascending order.

[z] Fisher's least significant difference ($P = 0.05$).

5B (growth chamber experiments), five BSR-resistant and four BSR-susceptible soybean genotypes were tested.

Field experiments (1A, 1B, 2A, and 2B). Field experiments were conducted on fields with a history of natural occurrence of BSR infestation. Plantings were done in 2 years, 2000 and 2001, at each of two locations, and the same sites were used in both years. One location was Curtiss Farm, located near Ames, (experiments 1A and 2A) planted on 16 May 2000 and 23 May 2001. The second location was at the NIACC center, near Mason City, (experiments 1B and 2B) planted on 24 May 2000 and 17 May 2001. The soil types were Clarion Loam and Clyde Silty Loam for Ames and Mason City locations, respectively. Plots were 1.5-m-long single rows, spaced 81 cm between rows, and planted with 20 seeds at a spacing of 7.6 cm between seeds. The experimental design used at each location and year was a complete randomized block with two replications per location.

BSR resistance/susceptibility of soybean lines was evaluated by growing plants on naturally infested soil and evaluating at the end of the growing season, at reproductive stages R7 to R8. Ten random plants were pulled from each plot. Stem length of each plant was measured from the soil line to the uppermost node, split longitudinally, and the height of internal stem discoloration was measured and expressed as a percentage of total stem length. No foliar symptoms were observed in field tests.

Greenhouse and growth chamber experiments (3A, 3B, 4A, 4B, 5A, and 5B). *Growth conditions.* Seeds of each soybean genotype were planted in a pasteurized mix of soil, sand, and perlite (2:1:2) in 4 × 21 cm plastic, cone-shaped containers (Stuewe and Sons, Corvallis, OR). There was one plant per container. In greenhouse experiments (3A, 3B, 4A, and 4B), plants were kept in a greenhouse set at constant temperature of 22°C; although actual mean temperatures ranged from 17 to 21°C depending on the experiment. In the growth chamber experiments 5A and 5B, plants were kept in a growth chamber with no humidity control and set at various temperature regimes as determined by the temperature treatment. In all greenhouse and growth chamber experiments, plants were grown under 16 h light and were fertilized weekly. In the greenhouse, high-pressure sodium lamps (400 W) were used to supplement natural light.

Inoculation. The *C. gregata* strain (OH2-3) used in these experiments was a single-spore isolate of strain OH2 provided by Cecil Nickell at the University of Illinois. Cultures were started on green bean extract (GBE) medium (35 g/liter ground frozen *Phaseolus vulgaris* L. green pods, 20 g/liter agar) supplemented with 50 mg/liter ampicillin. Cultures were incubated at room temperature (21 to 23°C) in the dark until abundant sporulation was visually evident. Conidia of *C. gregata* were suspended in 0.8% water agar (2.7×10^7 conidia/ml). The conidial suspension was thoroughly mixed (by tapping with sterile micropipette tips) into a paste. Stems of 2-week-old plants were punctured approximately 2 cm above the soil line with an 18-gauge needle (Becton Dickinson, Franklin Lakes, NJ) with its bevel filled with the inoculum paste. A needle with water agar paste without conidia was stabbed into stems of control plants.

Experimental design. Plants were arranged in a randomized complete block design; blocks were locations within a greenhouse or a growth chamber, and a treatment occurred once in each block. There were seven blocks in experiment 3A and nine in 3B. There were 10 blocks in each of experiments 4A and 4B, and four blocks in each of experiments 5A and 5B.

Foliar symptom assay. Foliar symptoms were assessed, 5 weeks after inoculation, as the proportion of symptomatic trifoliate leaflets divided by the total number of trifoliate leaflets. Each trifoliate was recorded as healthy, chlorotic, stunted, necrotic, or abscised. If a leaflet was normal in size, not deformed, and not necrotic, it was recorded as healthy or chlorotic depending on the predominant (>50%) color of the leaflet. If a leaflet was abnormally small and deformed but not necrotic, it was recorded as stunted. If there was any necrotic area in the leaf, it was recorded as necrotic, and if the leaflet had fallen off the plant it was recorded as abscised (missing). Severity of foliar symptoms was determined using the formula: (stunted trifoliate leaflets

Table 2. Brown stem rot resistance ratings of parents of populations 1 and 2 and resistant and susceptible checks using field and various greenhouse assays

Checks and parents with population 1[t]					Checks and parents with population 2[t]				
	Field[u]	Greenhouse[v,w]				Field[u]	Greenhouse[w,x]		
Experimental lines Cultivars	% Stem length discolored	% Stem length discolored	% Symptomatic leaflets	% Stem tips colonized	Experimental lines Cultivars	% Stem length discolored	% Stem length discolored	% Symptomatic leaflets	% Stem tips colonized
Parents[y]					Parents[y]				
A96-597011 (R)	6	74	24	50	A95-682026 (R)	12	55	22	15
A95-581022 (S)	32	77	26	42	P9233 (S)	11	56	62	65
Resistant checks					Resistant checks				
Archer	6	55	13	31	Archer	14	53	24	10
BSR101	9	69	15	13	BSR101	10	49	20	5
IA1006	8	41	12	7	IA1006	9	40	23	5
IA2008R	11	41	10	0	IA2008R	8	34	9	0
IA2050	7	71	16	14	IA2050	9	65	31	10
Susceptible checks					Susceptible checks				
Corsoy79	33	86	55	80	Corsoy79	38	87	88	85
IA2021	38	97	48	67	IA2021	23	78	84	85
IA3010	43	73	32	50	IA3010	33	63	59	75
Parker	19	76	42	47	Parker	15	67	60	80
Sturdy	21	80	50	75	Sturdy	18	86	76	74
LSD[z]	11	19	14	30	LSD[z]	13	15	17	21

[t] Data were analyzed using GLM in which soybean genotype is the only fixed effect and year, location, and replications (for field experiments) and replications and experiments (for greenhouse experiments) are treated as random effects.

[u] Numbers are percentage of stem length discolored and are means of two replications, each with 10 plants. Field experiments were conducted in 2 years, 2000 and 2001, at each of two locations, and the same sites were used in both years. One location was Curtiss Farm located near Ames, IA, planted on 16 May 2000 and 23 May 2001. The second location was at the NIACC center, near Mason City, IA, planted on 24 May 2000 and 17 May 2001.

[v] Numbers are means of seven and nine replications in each of experiments 3A and 3B; there is one plant in each replication.

[w] Stems of 2-week-old plants were punctured approximately 2 cm above the soil line with an 18-gauge needle with its bevel filled with the inoculum paste.

[x] Numbers are means of 10 replications in each of experiments 4A and 4B; there is one plant in each replication.

[y] (R) = BSR-resistant parent and (S) = BSR-susceptible parent.

[z] Fisher's least significant difference ($P = 0.05$).

Table 3. Pearson correlation coefficients (r) among brown stem rot resistance ratings using greenhouse, field, and molecular marker assays to evaluate progeny lines of population 1 and resistant and susceptible checks

		Progeny lines[w]		Parents and checks[w]	
	Assay[x]	Field stem discoloration, all years, all locations	Molecular marker[y]	Field stem discoloration, all years, all locations	Molecular marker
Greenhouse experiment[z]					
3A	Stem	0.26 a[z]	0.23 a[z]	0.79 a[z]	0.78 a[z]
	Foliar	0.60 b	0.55 b	0.76 a	0.86 a
	Fungus	0.64 b	0.76 c	0.66 a	0.81 a
	Fungus + foliar	0.69 b	0.77 c	0.70 a	0.84 a
3B	Stem	0.58 a	0.39 a	0.41 a	0.56 a
	Foliar	0.60 a	0.46 a	0.63 a	0.82 a
	Fungus	0.50 a	0.55 ab	0.59 a	0.65 a
	Fungus + foliar	0.66 a	0.64 b	0.66 a	0.80 a
3A and 3B	Stem	0.51 a	0.38 a	0.66 a	0.73 a
	Foliar	0.69 b	0.58 ab	0.70 a	0.87 a
	Fungus	0.65 ab	0.77 b	0.68 a	0.79 a
	Fungus + foliar	0.74 b	0.79 b	0.70 a	0.83 a
Field experiment[z]					
2000 Ames	Stem		0.40 a		0.66 a
2000 NIACC	Stem		0.68 a		0.87 b
2001 Ames	Stem		0.81 b		0.88 b
2001 NIACC	Stem		0.61 a		0.76 ab
All years, all locations	Stem		0.84 b		0.89 b

[w] Numbers are Pearson correlation coefficients for 50 progeny lines, 10 checks, and two parents; all coefficients are significantly ($P < 0.05$) different from zero. Pearson correlation coefficients between various assays were computed based on data from treatment means from each experiment and pooled experiment using the Corr procedure.

[x] Stem = % stem length discolored; foliar = % symptomatic trifoliate leaflets; fungus = % stem tips colonized; fungus + foliar assay is the average of foliar and fungus ratings.

[y] Numbers are computed based on seven and nine replications, one plant in each replication, in experiments 3A and 3B, respectively; two replications, each with 10 plants for individual field experiments.

[z] Within an experiment or pooled experiments, values with the same letters in a column are not significantly ($P < 0.05$) different from each other. Equality of correlation coefficients was tested using the modified Hotelling test. NIACC = Mason City, IA.

Table 4. Pearson correlation coefficients (r) among brown stem rot resistance ratings using greenhouse, field, and molecular marker assays to evaluate progeny lines of population 2 and resistant and susceptible checks

		Progeny lines[w]		Parents and checks[w]	
	Assay[x]	Field stem discoloration, all years, all locations	Molecular marker[y]	Field stem discoloration, all years, all locations	Molecular marker
Greenhouse experiment[z]					
3A	Stem	0.60 a[z]	0.53 a[z]	0.67 a[z]	0.69 a[z]
	Foliar	0.86 b	0.91 b	0.75 a	0.96 b
	Fungus	0.81 b	0.92 b	0.67 a	1.00 b
	Fungus + foliar	0.84 b	0.93 b	0.71 a	0.99 b
3B	Stem	0.49 a	0.47 a	0.56 a	0.61 a
	Foliar	0.72 b	0.81 b	0.65 ab	0.83 b
	Fungus	0.67 ab	0.75 b	0.81 b	0.88 b
	Fungus + foliar	0.76 b	0.85 b	0.75 b	0.88 b
3A and 3B	Stem	0.64 a	0.58 a	0.72 a	0.73 a
	Foliar	0.84 b	0.91 b	0.73 a	0.93 b
	Fungus	0.81 b	0.92 b	0.74 a	0.99 b
	Fungus + foliar	0.84 b	0.93 b	0.74 a	0.97 b
Field experiment[z]					
2000 Ames	Stem		0.64 ab		0.43 a
2000 NIACC	Stem		0.71 bc		0.70 a
2001 Ames	Stem		0.76 bc		0.73 a
2001 NIACC	Stem		0.47 a		0.66 a
All years, all locations	Stem		0.80 c		0.68 a

[w] Numbers are Pearson correlation coefficients for 50 progeny lines, 10 checks, and two parents; all coefficients are significantly ($P < 0.05$) different from zero. Pearson correlation coefficients between various assays were computed based on data from treatment means from each experiment and pooled experiment using the Corr procedure.

[x] Stem = % stem discolored; foliar = % symptomatic trifoliate leaflets; fungus = % stem tips colonized; fungus + foliar assay is the average of foliar and colonization rating.

[y] Numbers are computed based on 10 replications, one plant in each replication, in each of experiments 4A and 4B; two replications, each with 10 plants for individual field experiments.

[z] Within an experiment or pooled experiments, values with same letters in a column are not significantly ($P < 0.05$) different from each other. Equality of correlation coefficients was tested using the modified Hotelling test. NIACC = Mason City, IA.

+ necrotic trifoliate leaflets + abscised trifoliate leaflets/total trifoliate leaflets) × 100%. Chlorotic leaflets were not included in calculations of symptom severity because some of the noninoculated plants had chlorotic leaflets. Foliar symptoms were assessed without knowledge of the treatments.

Stem discoloration assay. Internal stem discoloration was assessed 5 weeks after inoculation, visually after splitting stems longitudinally. A stem was considered discolored if there was any visible dark brown discoloration on the vascular tissue or the pith of the stem. Severity of discoloration (percent stem length discolored) was calculated by dividing highest point of discoloration by total stem length × 100%. Because discoloration data are subjective, a single investigator collected all discoloration data within a block without knowledge of the treatment.

Fungal colonization assay. In experiments 3A, 3B, 4A, and 4B, fungal colonization was assessed once. In experiments 5A and 5B, it was assessed weekly over a 5-week period starting 1 week after inoculation. When it was done only once, assessment was made 5 weeks after inoculation, and only in the top 15% of stem tips. To determine the top 15% for cutting, the average plant height was determined by measuring the height of 20 randomly selected plants, and the length of stem tip for cutting in each plant was calculated relative to mean plant height. Stem tips were immersed for 2 min in 70% ethanol, rinsed in sterile, deionized water, and plated on GBE agar supplemented with ampicillin (50 mg/liter). In experiments in which *C. gregata* colonization was assessed weekly over a 5-week period (experiments 5A and 5B), stems were severed at the soil line and were immersed for 3 min in 70% ethanol, followed by 5 min in 10% sodium hypochlorite (commercial bleach) and a final rinse in sterile, deionized water. Stem length (plant height) was measured, and the stems were cut into 2-cm pieces. The relative position of each stem piece on the plant was recorded, and stem pieces were plated on GBE agar supplemented with ampicillin (50 mg/liter). The plates were incubated at 15°C in the dark for 15 days. The emerging fungal mycelia were examined microscopically for conidia and conidiophore morphology characteristic of *C. gregata*. A stem piece was considered colonized if *C. gregata* was recovered from any portion of the stem piece. In experiments 5A and 5B, stems were colonized starting from the base up to the maximum height colonized without gaps (data not shown). Consequently, severity of colonization was calculated by dividing the maximum height colonized by the total stem height multiplied by 100%.

Molecular marker assay (experiment 6). The 50 lines from each population, parents, and checks were also tested for the presence of marker 35E22.sp, a polymerase chain reaction (PCR) marker reported to be associated with BSR resistance (6). Young leaves were collected from two randomly selected plants in a plot planted to a progeny line from field plots in Ames, IA. The leaves were placed in a tube, immediately frozen in liquid nitrogen, and stored at –80°C. For DNA extraction, the leaves were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. DNeasy kit (Qiagen, Valencia, CA) was used to extract DNA. PCR primers, PCR reactions, restriction enzyme digestion, and electrophoresis were as described by Klos et al. (6) except that the amount of template DNA per PCR reaction varied from 10 to 30 ng/µl. Products of the PCR reaction were digested with restriction enzyme

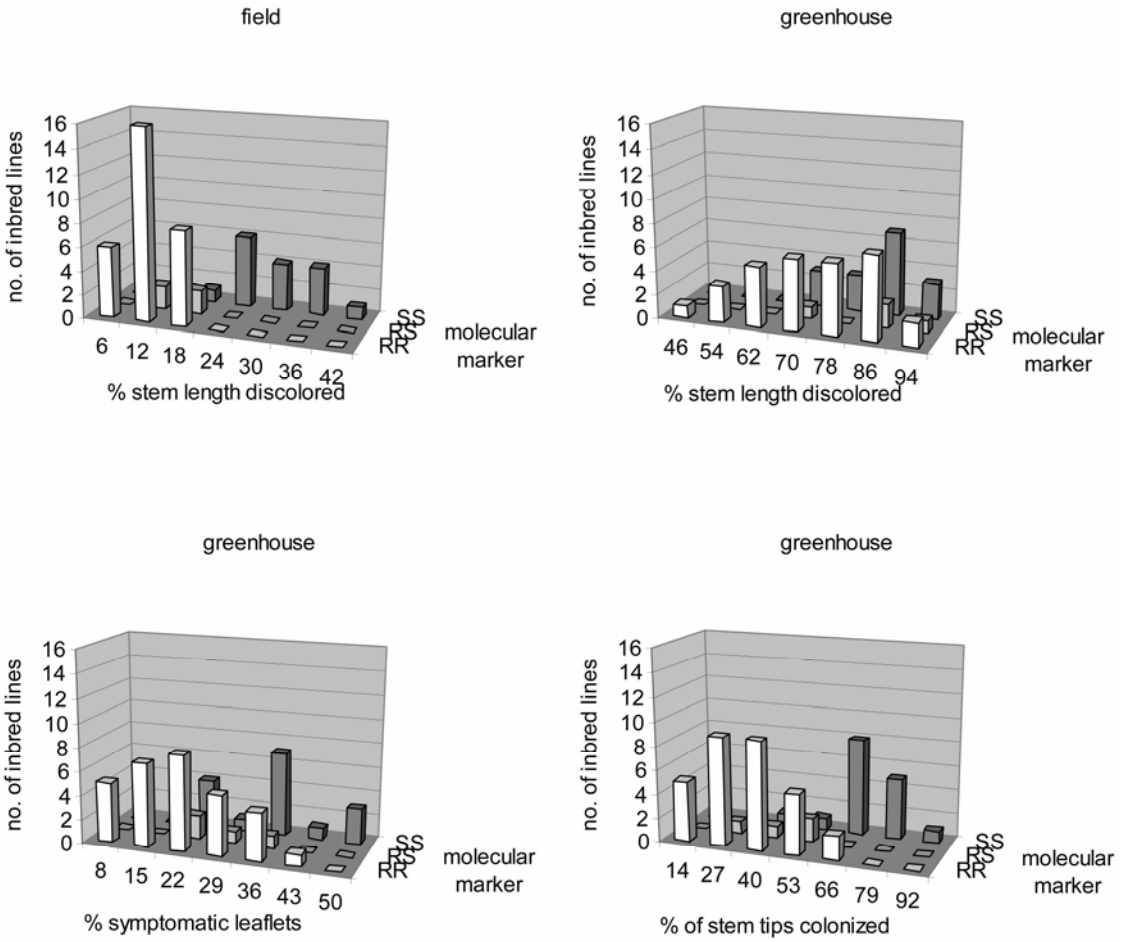

Fig. 1. Brown stem rot (BSR) severity ratings and the number of inbred lines of population 1 (n = 50) with the homozygous resistant (RR), heterozygous (RS), or homozygous susceptible (SS) alleles for BSR resistance molecular marker 35E22.sp. Field and several greenhouse BSR assays are compared for their ability to distinguish the lines (n = 50) with respect to the allele they possess. The "R" allele is derived from the resistant parent, and the "S" allele is derived from the susceptible parent.

*Hha*1 and run on 2% agarose gels in Tris-acetate-EDTA (TAE) buffer. Individual bands (alleles) were scored as homozygous resistant (RR) or susceptible (SS), or segregating (RS). The molecular marker assay was performed on each individual line at least twice using DNA from separate extractions. In a few cases, DNA extractions and PCR were performed up to four times to confirm the genotype of a line. If a line had inconsistent genotypes from the four PCR reactions, the line was assumed to be not totally inbred and considered heterozygous for the marker.

Data analysis. Statistical analyses were conducted using the SAS software package version 9.1. Data from field, greenhouse, and growth chamber experiments were analyzed using GLM in which soybean genotype is the only fixed effect and year, location, and replications (for field experiments) and replications and experiments (for greenhouse and growth chamber experiments) are treated as random effects (8). Pearson correlation coefficients between various assays were computed based on data from treatment means from individual experiments and pooled experiments using the Corr procedure. Equality of correlation coefficients was tested using the modified Hotelling test (16). Molecular marker data were tested for goodness of fit to a segregation ratio of 1 RR: 1 SS or 3 RR: 1 SS by chi-square test.

RESULTS

Field resistance screens (experiments 1A, 2A, 1B, and 2B). Differences in the severity of internal stem discoloration among the 50 lines within each population and among parents and checks were highly significant (Tables 1 and 2). In some experiments, the field resistance screen distinguished ($P < 0.05$) most of the resistant checks from most of the susceptible checks (data not shown). Nonetheless, in none of the experiments did the field assay distinguish every resistant check from every susceptible check, nor did pooled data across two locations and 2 years distinguish every resistant check from every susceptible check (Table 2). Data across locations and years distinguished a difference ($P < 0.05$) between the parents of population 1 but not between the parents of population 2 (Table 2).

Greenhouse resistance assays (experiments 3A, 3B, 4A, and 4B). Differences within each population and checks in the incidence of fungal colonization of stem tips and severity of foliar and stem symptoms were significant for both populations and parents and checks (Tables 1 and 2). Pooled data for experiments 3A and 3B and 4A and 4B suggest that the fungal colonization and foliar assays distinguished resistant from susceptible checks more clearly than did the stem discoloration assay (Table 2). The data also suggested significant ($P < 0.05$) differences in fungal colonization and foliar symptoms between the parents of population 2 (Table 2), but not between the parents of population 1 (Table 2).

Ability of greenhouse assays to predict field data. Data from the greenhouse experiments were significantly ($P < 0.05$) correlated (r) with stem discoloration data from field experiments for both populations, parents, and checks (Tables 3 and 4). For the checks and parents, there was no significant ($P < 0.05$) difference among greenhouse methods in how they correlated with field data except in experiment 4B (Table 4), in which colonization data were better correlated with field data than were greenhouse stem symptom data. For populations 1 and 2, greenhouse stem discoloration assays

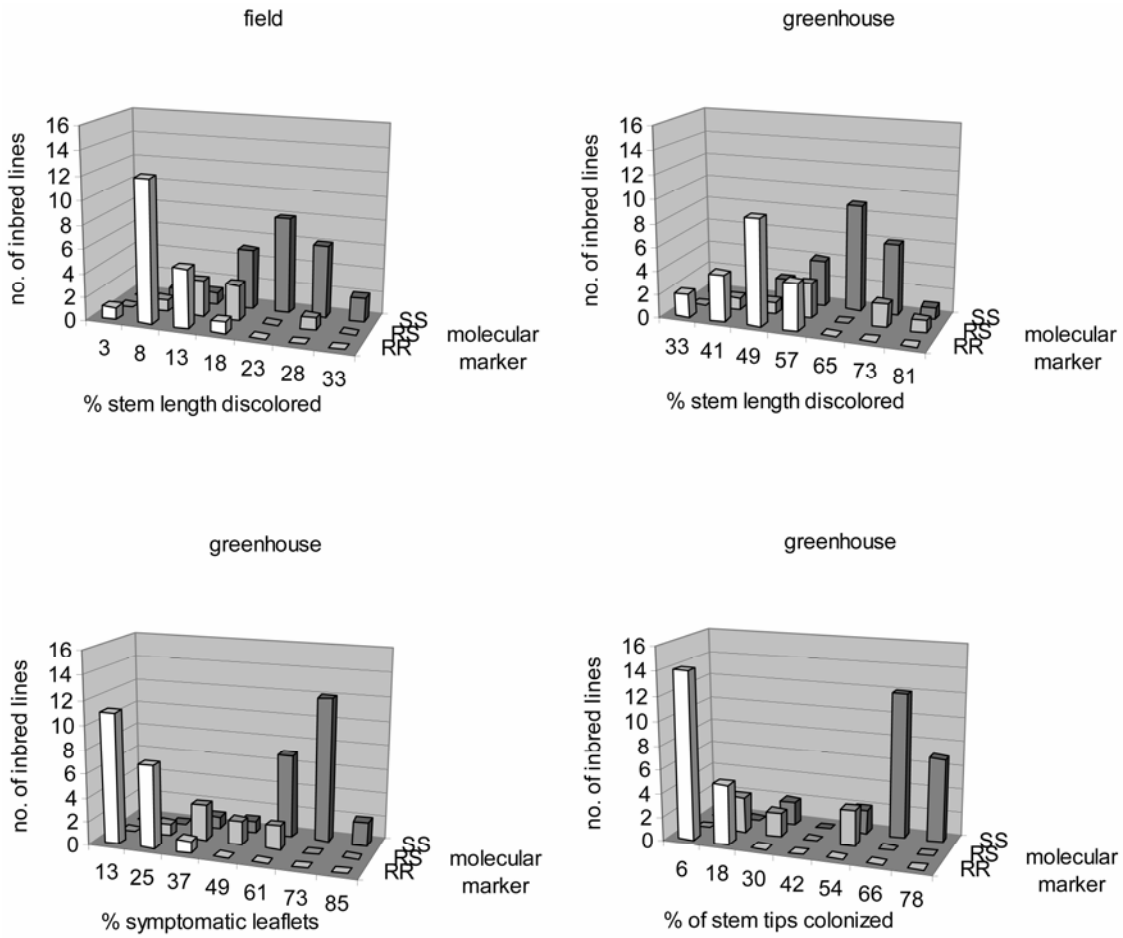

Fig. 2. Brown stem rot (BSR) severity ratings and the number of inbred lines of population 2 ($n = 50$) with the homozygous resistant (RR), heterozygous (RS), or homozygous susceptible (SS) alleles for BSR resistance molecular marker 35E22.sp. Field and several greenhouse BSR assays are compared for their ability to distinguish the lines ($n = 50$) with respect to the allele they possess. The "R" allele is derived from the resistant parent, and the "S" allele is derived from the susceptible parent.

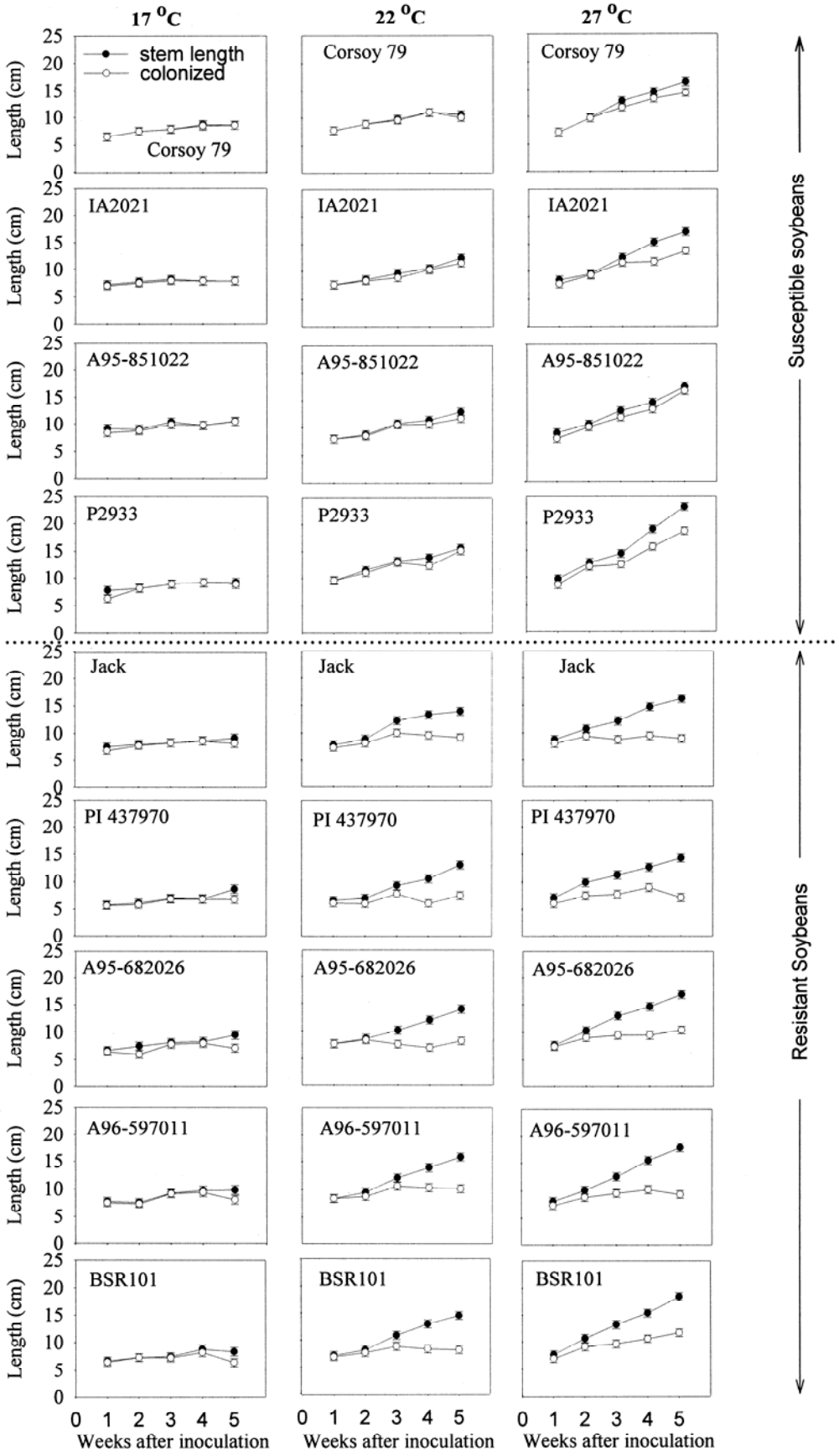

Fig. 3. Severity of stem colonization after introduction of conidia of *Cadophora gregata* into the base of stems of 2-week-old plants of four brown stem rot susceptible and five brown stem rot resistant soybean genotypes at three temperature regimes. Results are for combined experiments 5A and 5B. Each data point represents the mean of eight replicates with one plant each. Errors are experiment-wise standard errors.

mostly had the lowest correlation with field data (Tables 3 and 4).

For the two populations, linear regression analysis indicates that foliar symptoms explained the highest percentages of variability in field stem discoloration, followed by colonization and stem discoloration. The coefficients of determination (r^2, $P < 0.0001$) were 0.41 and 0.66 for colonization, 0.47 and 0.71 for foliar symptoms, and 0.25 and 0.40 for greenhouse stem symptoms, for population 1 and population 2, respectively. The high correlation between colonization and field assay and foliar assay and field assay suggest that these two greenhouse methods can be used for BSR screening in place of field assays.

Molecular marker assay (experiment 6). Segregation of molecular marker 35E22.sp was 30:4:16 for population 1 and 19:8:23 population 2 for homozygous resistant (RR), heterozygous (RS), and homozygous susceptible (SS) alleles of the marker, respectively. For population 1, 3.125% of the lines (1.56 lines) are expected to be heterozygous in the F_6 generation, and for population 2, which is in the F_5 generation, this percentage is 6.250 or 3.125 lines. In both populations, the number of heterozygotes was more than what was expected at their respective level of inbreeding. Assuming heterozygous lines will eventually segregate, in equal numbers, into homozygous RR and SS lines in generations beyond F_5 and F_6, the heterozygous lines were excluded from the chi-square test. For population 1, a 1 RR: 1 SS segregation was narrowly rejected ($\chi^2 = 4.26$ with 1 df, $P = 0.04$), while a 3 RR: 1 SS segregation was accepted ($\chi^2 = 1.86$ with 1 df, $P = 0.13$), suggesting two copies of the marker. For population 2, a 1 RR: 1 SS segregation was accepted ($\chi^2 = 0.38$ with 1 df, $P = 0.54$). These results suggest the marker is inherited as single locus.

Molecular marker data versus greenhouse and field data. For the two populations and checks, greenhouse data were significantly ($P < 0.05$) correlated with molecular marker data (Tables 3 and 4). Linear regression analysis suggested that fungal colonization data has a stronger relationship (r^2) with marker 35E22.sp than data from other greenhouse or field assays; greenhouse stem discoloration data has the lowest r^2 with the molecular marker. The r^2 ($P < 0.05$) values for populations 1 and 2, respectively, were 0.58 and 0.85 for colonization, 0.30 and 0.82 for foliar, and 0.11 and 0.33 for stem discoloration assays. The corresponding values for the field assays were 0.71 and 0.67. The above r^2 values were calculated based on pooled data from two experiments for greenhouse assays and four experiments for field assays.

Plotting greenhouse and field data in a frequency distribution indicated that the various BSR-resistance assays differed in their ability to clearly separate lines with identical molecular marker alleles into non-overlapping categories of BSR scores. Depending on the population, either foliar or colonization assays identified the highest number of homozygous resistant individuals into non-overlapping categories of BSR scores (Figs. 1 and 2).

Effect of temperature on fungal colonization (experiments 5A and 5B). Results from both experiments were similar, and consequently data were analyzed treating replications and experiments as random effects. The 22°C treatment was the best incubation temperature for separating BSR-resistant from BSR-susceptible soybeans. At 22°C, the fungus advanced closely with the growing stem (plant) tip of susceptible soybeans and lagged behind in resistant soybeans (Fig. 3); this difference between susceptible and resistant soybeans is the basis of our colonization assay. At 27°C, the fungus did not advance with the growing stem tip of some susceptible soybeans as closely as it did at 22°C (Fig. 3). At 17°C, the fungus advanced closely with the growing tip of both resistant and susceptible soybeans. The maximum difference between resistant and susceptible soybeans, as a group, in the percentage of stem length colonized by the fungus was detected in the 22°C treatment, 5 weeks after inoculation. These results suggest that fungal colonization assay is affected by temperature, and incubation at 22°C can be used to screen for BSR resistance using the colonization assay.

DISCUSSION

The fungal colonization method reported in this manuscript adds another option for screening soybeans for resistance to genotype A (2) of the BSR fungus, either in greenhouse or growth chamber conditions. The method employs inoculating the base of 2-week-old soybean plants with *C. gregata* followed by assessment of incidence of *C. gregata* colonization in the stem tips. In our experiments, the percentage of stem tips colonized was highly correlated both with BSR rating in the field and with the presence of a molecular marker (6) linked to BSR resistance (Tables 3 and 4).

Colonization and foliar assays were comparable in their effectiveness in distinguishing resistant from susceptible soybeans, although occasionally, one method was slightly more effective than the other. The combined colonization and foliar assays were consistently more correlated with field BSR and molecular marker data than each assay was alone (Tables 3 and 4). Thus, when a high-sensitivity BSR assay is a necessity, the colonization assay can be used in conjunction with a foliar assay if foliar symptoms develop.

The underlying difference in the movement of the fungus inside the stems of resistant and susceptible soybeans is the basis of our colonization assay. One week after introduction of the fungus into the base of 2-week-old seedlings, the fungus can be detected in the stem tips of both resistant and susceptible plants (Fig. 3). In subsequent weeks, the fungus colonizes the new apical stem growth at a faster rate in susceptible plants than in resistant plants (Fig. 3). Consequently, the fungus is isolated more frequently from the stem tips of susceptible than resistant plants. However, if new stem growth is insufficient, the fungus can be isolated frequently from the stem tips of both resistant and susceptible plants. On the other hand, if stem growth is too fast, the fungus might not be isolated from the stem tips of either susceptible or resistant plants. Thus, any environmental conditions that severely limit or accelerate new plant growth after inoculation may reduce the effectiveness of the colonization assay.

The greenhouse BSR resistance assays discussed in this manuscript can achieve reliable results in a short time and reduce the need for the typical multilocation and/or multiyear field screenings. The greenhouse assays using stem inoculation (i) reduce the incidence of disease escape compared with field or soil-infestation based methods, (ii) can be conducted year-round, shortening the time needed to develop BSR-resistant soybeans, and (iii) allow the optimization of environmental conditions for BSR development.

ACKNOWLEDGMENTS

This research was supported by Hatch Act (Project no. 3288) and State of Iowa funds, a grant from the Iowa Soybean Promotion Board, and a grant from the North-Central Soybean Research Program. We thank Philip Dixon, Department of Statistics, Iowa State University, for assistance in statistical analysis and our colleagues Mike D. Uphoff and David H. Soh for technical assistance.

LITERATURE CITED

1. Allington, W. B., and Chamberlain, D. W. 1948. Brown stem rot of soybean. Phytopathology 38:793-802.
2. Chen, W., Grau, C. R., Adee, E. A., and Meng, X.-Q. 2000. A molecular marker identifying subspecific populations of the soybean brown stem rot pathogen, *Phialophora gregata.* Phytopathology 90:875-883.
3. Harrington, T. C. and McNew, D. L. 2003. Phylogenetic analysis places the phialophora-like anamorph genus *Cadophora* in the Helotiales. Mycotaxon 87:141-151.
4. Harrington, T. C., Steimel, J., Workneh, F., and Yang, X. B. 2003. Characterization and distribution of two races of *Phialophora gregata* in the North-Central United States. Phytopathology 93:901-912.
5. Hughes, T. J., Chen, W., and Grau, C. R. 2002. Pathogenic characterization of genotypes A and B of *Phialophora gregata* f. sp. *sojae*. Plant Dis. 86:729-735.
6. Klos, K. L. E., Paz, M. M., Marek, L. F., Cregan, P. B., and Shoemaker, R. C. 2000. Molecular markers useful for detecting resistance to brown stem rot in soybean. Crop Sci. 40:1445-1452.
7. Lewers, K. S., Crane, E. H., Bronson, C. R., Schuup, J. M., Keim, P., and Shoemaker, R. C. 1999. Detection of linked QTL for soybean brown stem rot resistance in 'BSR101' as expressed in a growth chamber experiment. Mol.

Breed. 5:33-42.
8. Little, R. C., Wolfinger, R. D., Stroup, W. W., and Milliken, G. A. 1999. SAS system for mixed models. SAS Institute Inc., Cary, NC.
9. Nelson, R. L., Nickell, C. D., Orf, J. H., Tachibana, H., Gritton, E. T., Grau, C. R., and Kennedy, B. W. 1989. Evaluating soybean germ plasm for brown stem rot resistance. Plant Dis. 73:110-114.
10. Phillips, D. V. 1971. Influence of air temperature on brown stem rot of soybean. Phytopathology 61:1205-1208.
11. Sebastian, S. A., and Nickell, C. D. 1985. Inheritance of brown stem rot resistance in soybeans. J. Hered. 76:194-198.
12. Sebastian, S. A., Nickell, C. D., and Gray, L. E. 1985. Efficient selection for brown stem rot resistance in soybeans under greenhouse screening conditions. Crop Sci. 26:753-757.
13. Tabor, G. M., Tylka, G. L., Behm, J. E., and Bronson, C. R. 2003. *Heterodera glycines* infection increases incidence and severity of brown stem rot in both resistant and susceptible soybean. Plant Dis. 87:655-661.
14. Tabor, G. M., Tylka, G. L., Cianzio, S. C., and Bronson, C. R. 2003. Resistance to *Phialophora gregata* is expressed in the stems of resistant soybeans. Plant Dis. 87:970-976.
15. Tachibana, H., Voss, B. K., and Fehr, W. R. 1987. Registration of 'BSR101' soybean. Crop Sci. 27:612.
16. Williams, E. J. 1959. Regression Analysis. John Wiley & Sons Inc., New York.
17. Wilmot, D. B., and Nickell, C. D. 1989. Genetic analysis of brown stem rot resistance in soybean. Crop Sci. 29:672-674.
18. Workneh, F., Tylka, G. L., Yang, X. B., Faghihi, J., and Ferris, J. M. 1999. Regional assessment of soybean brown stem rot, *Phytophthora sojae*, and *Heterodera glycines* using area-frame sampling: Prevalence and effects of tillage. Phytopathology 89:204-211.
19. Yang, X. B., and Lundeen, P. 1997. Soybean brown stem rot. Iowa State University, Ext. Publ. Pm-890.